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Review

Metallocarboxypeptidases and their protein inhibitors Structure, function and biomedical properties

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Abstract

Among the different aspects of recent progress in the field of metallocarboxypeptidases has been the elucidation of the three dimensional structures of the pro-segments (in monomeric or oligomeric species) and their role in the expression, folding and inhibition/activation of the pancreatic and pancreatic-like forms. Also of great significance has been the cloning and characterization of several new regulatory carboxypeptidases, enzymes that are related with important functions in protein and peptide processing and that show significant structural differences among them and also with the digestive ones. Many regulatory carboxypeptidases lack a pro-region, unlike the digestive forms or others in between from the evolutionary point of view. Finally, important advances have been made on the finding and characterization of new protein inhibitors of metallocarboxypeptidases, some of them with interesting potential applications in the biotechnological/biomedical fields. These advances are analyzed here and compared with the earlier observations in this field, which was first explored by Hans Neurath and collaborators.

Keywords: Metallocarboxypeptidase; Pro-carboxypeptidase; Zymogen activation; Protein inhibitor; Epidermal growth factor antagonist

1. Metallocarboxypeptidases

Carboxypeptidases are exopeptidases that catalyze the hydrolysis of peptide bonds at the C-terminus of peptides and proteins. This enzymatic action may be carried out by at least two different kinds of enzymes with different catalytic mechanisms: metallocarboxypeptidases, which possess a tightly bound Zn^{2+} atom directly involved in catalysis, and serine-carboxypeptidases, which contain a reactive Ser residue at the active site that belongs to the Ser/His/Asp triad characteristic of serine proteinases. Among metallocarboxypeptidases, the most thoroughly studied group of enzymes is that of family M14 in the Barrett-Rawlings-Woessner classification [1]. This review will focus on the description of some members of this family and their protein inhibitors.

Depending on the criteria used, carboxypeptidases can be classified in different ways. For instance, metallocarboxypeptidases are not homogeneous with regard to their zinc-binding motifs and can be classified into distinct groups based upon their zinc binding site [2,3]. Among the members of family M14, carboxypeptidases may also be classified upon the basis of their substrate specificity. Carboxypeptidase A-like enzymes have a preference for hydrophobic C-terminal residues, and B-like enzymes cleave only

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the C-terminal basic residues Lys or Arg. The A forms can be subdivided into the A1 and A2 isoforms, the latter exhibiting a preference for C-terminal bulky aromatic residues [4–6]. Two subfamilies of M14 can be defined based upon sequence homology and overall structure: those of carboxypeptidase A, the structural prototype for most metallocarboxypeptidases, and carboxypeptidase E/H. The degree of amino acid identity is greater than 40% within each subfamily and about 15–20% between subfamilies. The two groups also differ in the length of their enzyme domains, which is about 310 residues for the members of the carboxypeptidase A subfamily and more than 100 residues longer for the members of the carboxypeptidase E/H subfamily. The only examples of multisubunit or multicatalytic domain structures are found within the members of the latter subfamily, which frequently appear to be glycosylated.

An alternative subdivision is also possible based on the involvement of the enzymes in specific physiological processes: pancreatic carboxypeptidases A1, A2 and B typically function only as digestive enzymes, whereas the rest of mammalian carboxypeptidases, including carboxypeptidase A from mast cells and carboxypeptidase U (or plasma carboxypeptidase B), exert their action in various physiological processes, mainly in non-digestive tissues and fluids, and have been called 'regulatory carboxypeptidases' because of their involvement in more selective processing reactions [7].

1.1. Structure and catalytic mechanism

Carboxypeptidase A is the prototype zinc peptidase, and it was the third protein and the first Zn^{2+} metalloenzyme for which a high resolution structure was obtained [8]. The current knowledge on the structure, enzymatic action and general behavior of carboxypeptidases results, to a significant extent, from the excellent seminal work of Hans Neurath, William N. Lipscomb and Bert L. Vallee and their collaborators.

Crystal structures are now available for pancreatic pro- and carboxypeptidases A1, A2 and B [9–15] and for other metallocarboxypeptidases such as carboxypeptidase T [16], G2 [17] and Zinc-D-Ala-D-Ala carboxypeptidase [18]. No three-dimensional (3-D) structure has yet been reported for a member of the regulatory group. However, an essential overall similarity must exist between the active sites of pancreatic-like and regulatory carboxypeptidases since they all share a zinc binding motif of the type **HXXE...H**, where the three zinc ligands are a histidine, a glutamate located two residues downstream and a second histidine located 108–135 amino acids further to the C-terminus from the first histidine. They all conserve the residues that are essential for activity at similar locations in the sequence.

Crystallographic and kinetic studies of complexes of bovine carboxypeptidase A1 with natural or synthetic inhibitors [19–23] and kinetic studies performed on recombinant mutant forms [24,25] have made this enzyme one of the most thoroughly studied, and often used as an example of catalytic mechanism in textbooks. However, a controversy still subsists between the two hypotheses launched to explain the catalytic mechanism of the enzyme [8,26]. The acyl pathway hypothesis proposes the existence of a covalent acyl enzyme intermediate whereas the promoted-water pathway requires a simultaneous polarization of the substrate carbonyl group by the zinc ion and a zinc-promoted activation of a water molecule, which directly attacks the scissile peptide bond [21]. Arguments are currently in favor of the promoted-water pathway [27,28] where a pentacoordinated zinc polarizes a water molecule to attack the scissile peptide bond of the substrate, leading to a tetrahedral intermediate/transition state.

The zinc binding residues are His-69, Glu-72 and His-196 (numbering of carboxypeptidase A1). From crystallographic studies a number of amino acids have been defined as important for substrate binding and catalysis and can be classified in several subsites: Asn-144, Arg-145, Tyr-248 in S1′; Arg-127 and Glu-270 in S1; Arg-71, Ser-197, Tyr-198 and Ser-199 in S2; Phe-279 in S3 and Glu-122, Arg-124 and Lys-128 in S4. The terminal carboxylate group of the peptide substrate is fixed by Asn-144, Arg-145 and Tyr-248, while the carbonyl group of the scissile peptide bond is positioned near Glu-270, Arg-127 and the zinc. All these residues are conserved among digestive carboxypeptidases and many of them in regulatory carboxypeptidases, the most important differences being restricted to the amino acids that define specificity of substrate or are located at surface regions.

1.2. A variety of functions: digestive and regulatory carboxypeptidases

The hydrolysis of peptide bonds at the C-terminus of peptides and proteins carried out by carboxypeptidases may be a step in the degradation of some substrate molecules or result in the maturation of others. The physiological effect of these enzymes, as for every type of protease, is thus varied and site- and organism-dependent. Two main groups of carboxypeptidases can be defined in family M14 with respect to the type and location of their physiological functions: the digestive (or pancreatic) carboxypeptidases, whose primary function is to act on the degradation of intake proteins, and the regulatory carboxypeptidases, whose substrates are normally biologically active peptides in non-digestive tissues and fluids. Most of the members of the latter group are also called 'basic' carboxypeptidases because they are usually specific for C-terminal basic residues.

Carboxypeptidase A from mast cells and carboxypeptidase U (or plasma carboxypeptidase B) are the two regulatory carboxypeptidases that are closer in structure to the pancreatic ones, with a degree of sequential identity greater than 40%. They are the only proteins within this group that are synthesized with a pro-peptide of the same length as the pancreatic forms. The first one is found in the secretory granules of mast cells, where it forms macromolecular complexes with proteoglycans. Granule-bound carboxypeptidase A is fully active [29,30] and cleaves peptide substrates in tandem with other proteases. The precise role of this carboxypeptidase is still to be defined, although it may obviously be related with pathological conditions in which mast cells have been implicated, like allergic responses, inflammation and others [31]. Carboxypeptidase U, a carboxypeptidase B-like enzyme from plasma, is found in blood as a zymogen [32] and has been defined as a thrombin-activable fibrinolysis inhibitor [33]. Its activation is in fact modulated by the thrombin-thrombomodulin complex where the epidermal growth factor-like domain 3 of the latter seems to play an essential role [34]. Because of its affinity for basic C-terminal residues, carboxypeptidase U may release lysines at the binding sites for plasminogen on the surface of cells and on fibrin, and seems to modulate plasminogen activation and the rate of fibrinolysis.

The other regulatory carboxypeptidases do not possess an inactivating propeptide like those of the forms previously commented upon, although carboxypeptidase E is synthesized with a N-terminal 14-residue propeptide which is well conserved among species [35]. Carboxypeptidases E and D are involved in the processing of biologically active peptides and their distribution indicates that the number of their possible peptide substrates is very large [36–38]. This is even more clear in the case of carboxypeptidase D, found in almost all tissues examined [39]. Carboxypeptidase D is the sole member of the regulatory group which occurs as a multidomain (but single-chain) protein. It has three carboxypeptidase domains, the third one being catalytically inactive [37,40]. Met- and Leu-enkephalin, insulin, vasopressin and many others are peptide substrates for carboxypeptidase E, while proteins that are secreted via the constitutive pathway, such as growth factors and growth factor receptors, are likely to be substrates for carboxypeptidase D [39].

Carboxypeptidase M was first purified and characterized from human placental microvilli [41]. It is a widely distributed membrane-bound ectoenzyme which is found at high levels in placenta and lungs and also in a variety of tissues and cells. Carboxypeptidase M can be solubilized after release of its hydrophobic anchor to the membrane by treatment with phosphatidylinositol-specific phospholipase C and trypsin [42]. The localization of carboxypeptidase M in the plasma membrane indicates that it could participate in the control of peptide hormone activity at the cell surface, in the modulation of receptors and in extracellular protein processing or degradation. Among other processes, carboxypeptidase M has been implicated in the metabolism of growth factors from its capacity for generating des-Arg53-EGF [43] and the coincidence of distribution of carboxypeptidase M and epidermal growth factor. The enzyme could also be involved in protective functions at the alveolar surface and in inflammatory and other pathological processes.

Carboxypeptidase N is the largest regulatory carboxypeptidase described. It is synthesized in the liver and released in the blood where it is present at high concentrations (30 $\mu g\ ml^{-1}$). It cleaves an ample variety of peptide and protein substrates and is the major blood-borne inactivator of potent peptides

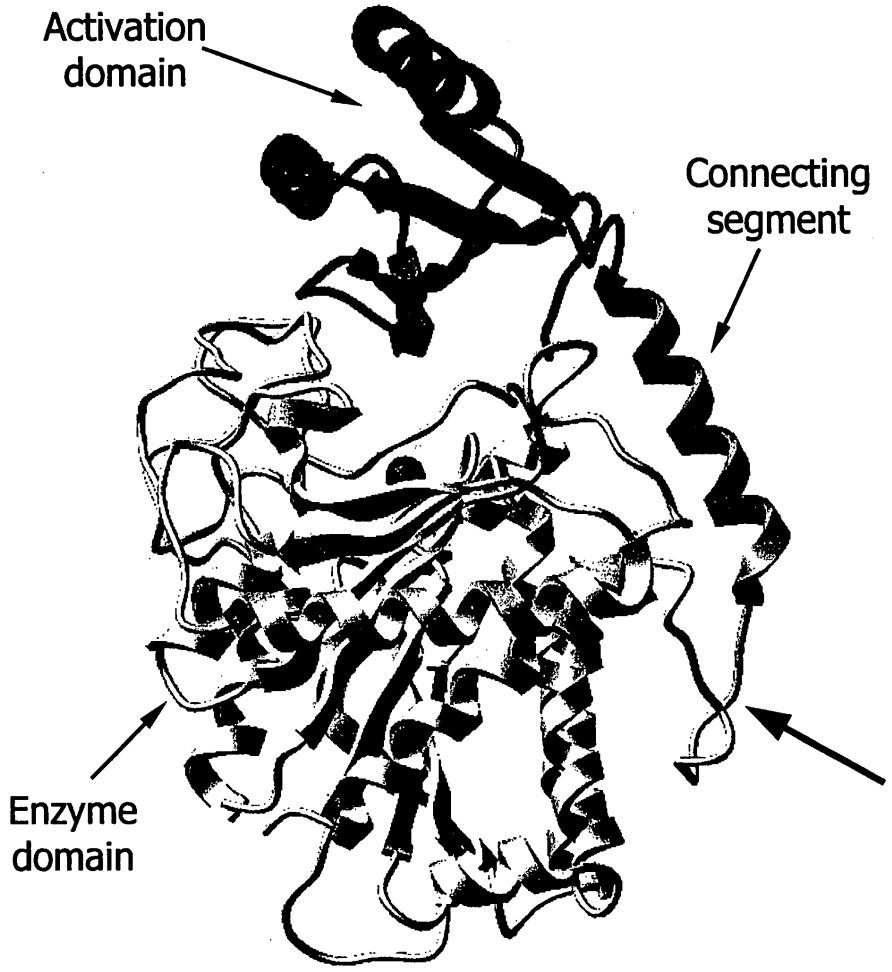

Fig. 1. Ribbon plot of the 3-D structure of pancreatic procarboxypeptidase A1. The single-polypeptide zymogen is represented in a gray scale to highlight the different conformational elements. Light gray: enzyme domain; medium gray: connecting segment; dark gray: activation domain. The last two elements form the activation segment (or pro-segment) which is scissed after tryptic activation.

such as kinins and anaphylotoxins. It is believed that carboxypeptidase N has a general protective function in blood [7], as shown by a number of clinical studies. To be fully active, carboxypeptidase N needs the presence of the catalytic (50–5 kDa) subunit and the accompanying glycosylated 83 kDa subunit [44]. The latter protects the former from degradation and also impedes its removal from the blood by glomerular filtration [45].

Other members of the regulatory carboxypeptidase subfamily include carboxypeptidases Z [46], X1 [47] and X2 [48]. Transcripts of pancreatic carboxypeptidases have been detected in extrapancreatic tissues, including brain [49], where they appear to be the product of alternative splicing. The continuously increasing knowledge about the variety of functions in which carboxypeptidases are involved makes of this field of research a very dynamic one.

The biomedical and biotechnological applications of carboxypeptidases are currently being explored. A few but significant reports describe the use of human carboxypeptidases to produce enzymes with a reversed specificity [50] or to generate enzyme-antibody conjugates used in the antibody-directed enzyme prodrug therapy (ADEPT) [51,52]. A deep knowledge of the structure of human carboxypeptidases and of the effects of their redesign are needed to generate products of therapeutic value.

1.3. Procarboxypeptidases: general features and oligomeric association

Pancreatic and pancreatic-like carboxypeptidases are synthesized as inactive precursors or zymogens. As for the active enzymes, the pro-carboxypeptidases were among the first zymogens to be studied and characterized through the pioneering work of Hans Neurath and collaborators [53,54]. Procarboxypeptidases are peculiar among pancreatic proenzymes in that their pro-peptides are remarkably long: they may vary from 94 to 96 residues, which accounts for about one-fourth of the molecule. The synthesis of proteases in the form of a zymogen is a well-known control mechanism that avoids the deleterious effects of placing such enzymes at improper times or locations and permits release of their activities only after specific limited proteolysis [55]. In this particular case, however, the pro-peptides or activation segments, besides maintaining the enzyme inactive in the pre-secretional phase, also act in some cases as a post-activation mechanism of control (see further on).

The detailed 3-D structures of monomeric procarboxypeptidases A1, A2 and B have been determined [12,13,15]. The structure of the enzyme moiety in the zymogens is almost identical to the structure found in the isolated active enzymes [10,56]. In the three cases, the activation segment adopts the structure of a globular domain that covers the wide active site depression of the enzyme (Fig. 1). Besides the monomeric forms found in most species, procarboxypeptidases also occur either as non-covalent binary complexes with proproteinase E or chymotrypsinogen C or as ternary complexes with the two serine proproteinases; the occurrence of the different oligomeric complexes is species-dependent. It exists in ruminants where the balance is shifted towards the formation of ternary complexes [57–59]. It is interesting to note that proproteinase E (now pancreatic endoproteinase E [1]) was described for many years as a N-terminal truncated form called subunit III that could not be activated by trypsin because of the lack of the N-terminal dipeptide essential for the definition of the active site [60]. However, it was subsequently shown that such a truncation is not present in the native form of the molecule but is artificially produced during the isolation [61].

The arrangement of the subunits in the bovine ternary complex explains the decreased rate of proteolytic activation of the procarboxypeptidase A subunit, a phenomenon also observed with binary complexes [62,63]. It is a combined effect of a decreased accessibility of the tryptic activation targets and the stability of the complex that leads to the slow activation rate observed. The resolution of the 3-D structure of the native ternary complex [14,64] confirms this view, showing that the activation region of procarboxypeptidase A is in contact with the other two subunits (Fig. 2) making the access of both the activating protease and the substrates difficult. On the other hand, the activation sites of the other two serine proproteinases are exposed to the solvent and freely accessible to proteolytic attack. The oligomeric organization of the complex probably has a functional role and suggests a sequential activation of the different subunits. The bovine ternary complex is thus an excellent example of the correlation between quaternary structure and biological activity: while the association with the two serine proproteinases hinders limited proteolysis of procarboxypeptidase A, the activation segment both prevents carboxypeptidase A activity expression and maintains the complex stable until its trimming has been extensive enough, leading to the release of all the mature monomeric enzymes.

2. The pro-segments: autologous inhibitors

Pancreatic-like metallocarboxypeptidases are kept inactive until their action is needed by the presence of a long N-terminal activation segment, pro-region or pro-peptide. The activation segments are autologous protein inhibitors of carboxypeptidases but they are not the only known protein inhibitors of such enzymes.

2.1. From N-terminal tails to intramolecular chaperones

The initial reports on procarboxypeptidases did not pay much attention to the structure of the pro-segments and the functional implications of their presence in the newly synthesized zymogen partially due to their difficult isolation and handling, although

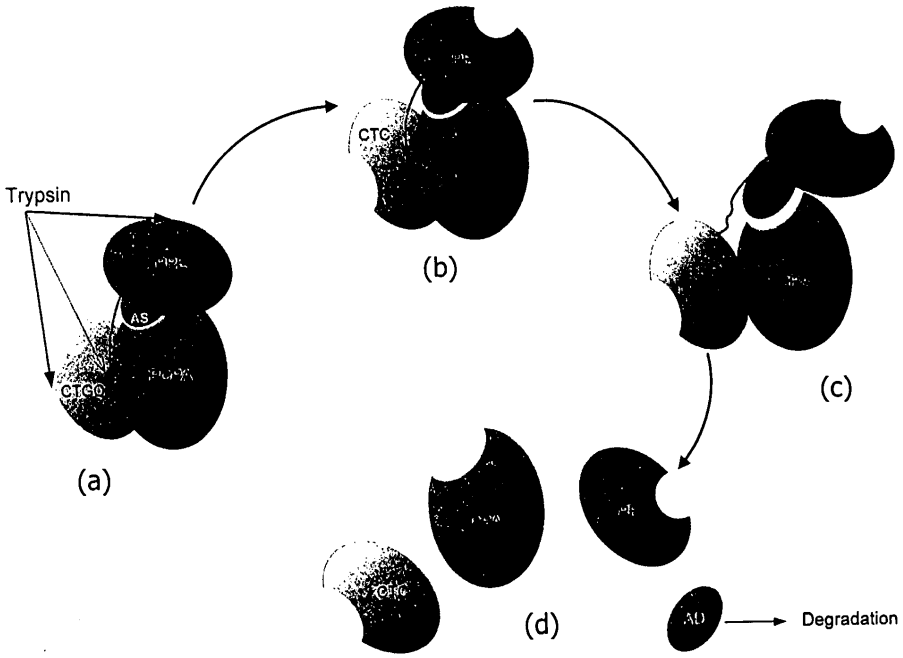

Fig. 2. A scheme for the activation of the ternary complex of bovine procarboxypeptidase A1. (a) Trypsin is able to readily cleave the exposed activation targets of chymotrypsinogen C (CTGC) and proproteinase E (PPE) while most of the procarboxypeptidase A (PCPA) remains uncleaved due to the maintenance of the ternary complex even if some cleavage has occurred at the almost inaccessible primary scissile peptide bond of PCPA. The strong binding between CPA and its activation segment (AS) is basic for the stability of the ternary complex. (b and c) The initially slow action of trypsin on the PCPA primary target releases a slowly increasing population of molecules with destabilized quaternary structure that finally leads to (d) Cleavage of the secondary tryptic targets, disassociation of the complex, release of full CPA activity and full degradation of the activation domain (AD), the globular part of the activation segment.

the pioneering work of Neurath and co-workers [62,65] and other groups [66,67] had defined the basis for their study.

The fate of the pro-peptides after tryptic activation of the pancreatic zymogens is, in the long term, their degradation to the constituent amino acids. However, some reports point out the presence of the pro-segment of pancreatic carboxypeptidase B in urine in cases of acute pancreatitis and describe a relatively slow turnover rate [68,69]. The isolation of pro-segments from a protease-rich medium proved to be difficult; the first report on their structure dates from 1982 [70]. The globular parts of the pro-segments of pancreatic carboxypeptidases were found to be independently folding units of remarkable stability. More recent reports have discussed the properties of the activation segments, not only as modulators of the activation processes, but also as important elements for the proper folding of the enzyme moiety. Thus, it has been observed that the presence of this fragment greatly facilitates the heterologous expression of the proenzymes [71,72], with the pro-segment probably assisting in the folding of pancreatic carboxypeptidases in vivo. Indirect evidence has also been obtained from site-directed mutagenesis studies in which mutations affecting relevant residues in the contact zone of enzyme and pro-segment moieties completely abolish secretion of the recombinant [71]. In the case of yeast serine carboxypeptidase Y, that belongs to a different family, the pro-segment acts as a cotranslational chaperone [73]. Although the structures of metallo and serine carboxypeptidases are not readily comparable, this observation confirms the high folding capability of pro-segments in heterologous systems, as shown for other proteases [74].

2.2. *The pro-segments are inhibitory globular domains*

The original reports on the structure and behavior

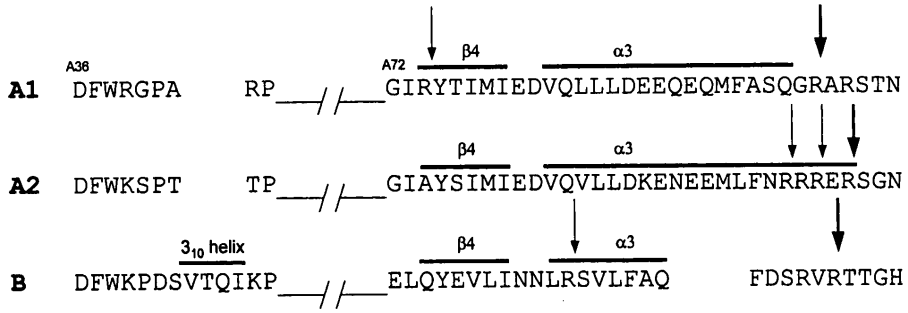

Fig. 3. Comparison of the sequences of regions in the activation segments of procarboxypeptidases that interact with the enzyme moiety and affect activity and activation. The sequences correspond, from top to bottom, to the A1, A2 and B forms. The regions around residue 40 (left) and the C-terminus of the activation segment (right) are shown; the first residues of every sequence segment (A36 and A72) are indicated; in this notation A refers to the convention used in the PDB to indicate that such residues belong to the activation segment. Above the sequences, the type and size of the secondary structure elements are shown. Strand β4 and helix α3 are present in all three sequences, whereas only the B form possesses a 3_{10} helix turn. Thick and thin arrows indicate the location of, respectively, the primary and the secondary trypsin targets during activation.

of pro-segments showed that these pieces behave as potent (in the nM range) inhibitors of the enzymes in the isolated state [75]. However, only the pro-segments isolated from the A forms of procarboxypeptidases show this property; the isolated segments of the B forms are not inhibitory in *trans* [76,77]. The pro-segments, however, do behave as inhibitors of carboxypeptidases before trypsin activation occurs, rendering the active center inaccessible to protein and peptide substrates. Moreover, procarboxypeptidases B are absolutely devoid of intrinsic activity while the A forms are able to catalyze the hydrolysis of small peptide substrates, probably by diffusion through a channel between the pro-segment and the enzyme that permits limited access to the active center.

The sequence identity between the pro-segments of digestive metallocarboxypeptidases varies from 54% between the A1 and A2 forms to 27% and 22% when compared with the B form (for sequences see Fig. 3). However, an overall identity in folding topology is observed in all the proteins studied so far. 3-D crystal structures are available for the A1 and B porcine zymogens [12,13] and for the human A2 zymogen [15], and the structure of the activation domain of porcine procarboxypeptidase B has been solved by NMR [78]. The pro-segments of all these forms fold as an open sandwich antiparallel α/antiparallel β core followed by an extended α helix at the C-terminus which connects it to the enzyme moiety. The globular core of the pro-segment covers the active site of the enzymes, shielding the S2, S3 and S4 subsites. In addition, Arg-145 (at S1′ in the enzyme moiety) is directly bound to Asp-41 from the pro-segment in the B proenzyme, a link that is likely to strongly contribute to the absolute lack of intrinsic proteolytic activity of this form.

The globular core of the pro-segments of procarboxypeptidases, or activation domain, is a single polypeptide of about 80 residues with no disulphide bridges and constitutes a good model for studies on protein folding and redesign. One such piece, ADA2h (activation domain of human procarboxypeptidase A2), has been extensively studied by site-directed mutagenesis with the aim of addressing questions on protein folding and stability and also to get information about the structural determinants for the improvement of the inhibitory action of such molecules. It has been observed that ADA2h follows a two-state transition in the folding process with no intermediates [79] and that the initially high stability of the protein can be further increased by the improvement of the propensities of helix formation through site-directed mutagenesis of selected residues in the sequences that form helical structures [80,81]. The transition state of the folding process has also been analyzed by protein engineering and shown to be quite compact and with some degree of secondary structure [82]. Interestingly, it has also been observed that helix redesign may reverse the α-helix to β-sheet

transition that occurs with the wild type protein in certain conditions (V. Villegas, personal communication).

2.3. Controlled degradation of pro-segments: the activation of pancreatic-like procarboxypeptidases

Although pancreatic procarboxypeptidases have very similar 3-D structures and share the same basic inhibition mechanism - a globular activation domain covalently linked to the enzyme moiety that shields the active site and maintains the molecule inactive until the covalent link is broken - their activation pathways and rates appear to be very different [77,83,84]. Despite the higher identity between A1 and A2, the A1 form shows a slow, biphasic, activation pathway as compared to both the A2 and B forms which have a faster, monotonic, activation behavior. On the other hand, only the B form is absolutely devoid of intrinsic activity in the zymogen state.

The activation domains of pancreatic procarboxypeptidases have shown to be stable, globular entities, as described above. Therefore, carboxypeptidases will be inhibited as long as the activation domain of the pro-segment is kept in place. From X-ray diffraction studies, two regions of the pro-segments are clearly responsible for the interaction with the enzyme: the globular activation domain and the connecting region that covalently links both globular moieties (see Fig. 1).

Both regions show significant differences when the A and B forms are compared. The connecting region is longer and more structured in A1 and A2 while it is shorter and with poor local secondary structure in the B form (see Fig. 3). This difference can explain the extremes in activation rate shown by A1 and B: it could be envisaged that the first activating event perturbs the limited local stability of the connecting region and promotes its release from the enzyme surface, facilitating the tryptic attack on the secondary target, which happens to be more accessible in the B form. From this it could be concluded that the length and stability of the connecting region are the main factors responsible for the binding of the pro-piece to the enzyme after the initial tryptic cleavage. However, that explanation does not fit with the observations made with the A2 form, where, despite the presence of a connecting region even longer and better structured than in the A1 form, the activation behavior is closer to that shown by the B form. In this case the conclusion drawn from the comparison between A1 and A2 is just the opposite: it would seem that the different velocities of the activation processes are mainly determined by the inhibitory capability of the activation domain once cut from the proenzyme.

This apparent contradiction suggests that the inhibition/activation mechanism of metallo-procarboxypeptidases is the joint result of a number of factors. Calculations of surface complementarity and interaction energies have shown that the buried surface between the pro-segment globular domain and the enzyme is larger in A1 [85] and also that the electrostatic energy is less favorable in the case of A2, followed by B and A1 [86]. Thus, both contact areas are necessary, and none of them sufficient, to maintain the zymogen state of procarboxypeptidases as has also been lately shown by site-directed mutagenesis studies on the B form [87].

3. Heterologous protein inhibitors

In contrast to endoproteases, for which numerous cases have been reported, polypeptides that specifically inhibit metallocarboxypeptidases have only been found in potatoes, tomatoes, roundworms, leech and in some mammalian tissues [49,88–90]. The most extensively studied of these inhibitors is the potato carboxypeptidase inhibitor (PCI or CPI) which was the first to be characterized by the pioneer work of Neurath, Ryan and collaborators.

3.1. Potato carboxypeptidase inhibitor: from standard protein engineering studies to EGF antagonists and cancer

The carboxypeptidase inhibitor from potato, a 39-residue protein, is among the smallest globular proteins described. The 3-D structure is known in aqueous solution [91], and as a crystal complex with carboxypeptidase A [20]. The fold of the inhibitor is depicted in Fig. 4. The 27-residue globular core of PCI is stabilized by three disulphide bridges and

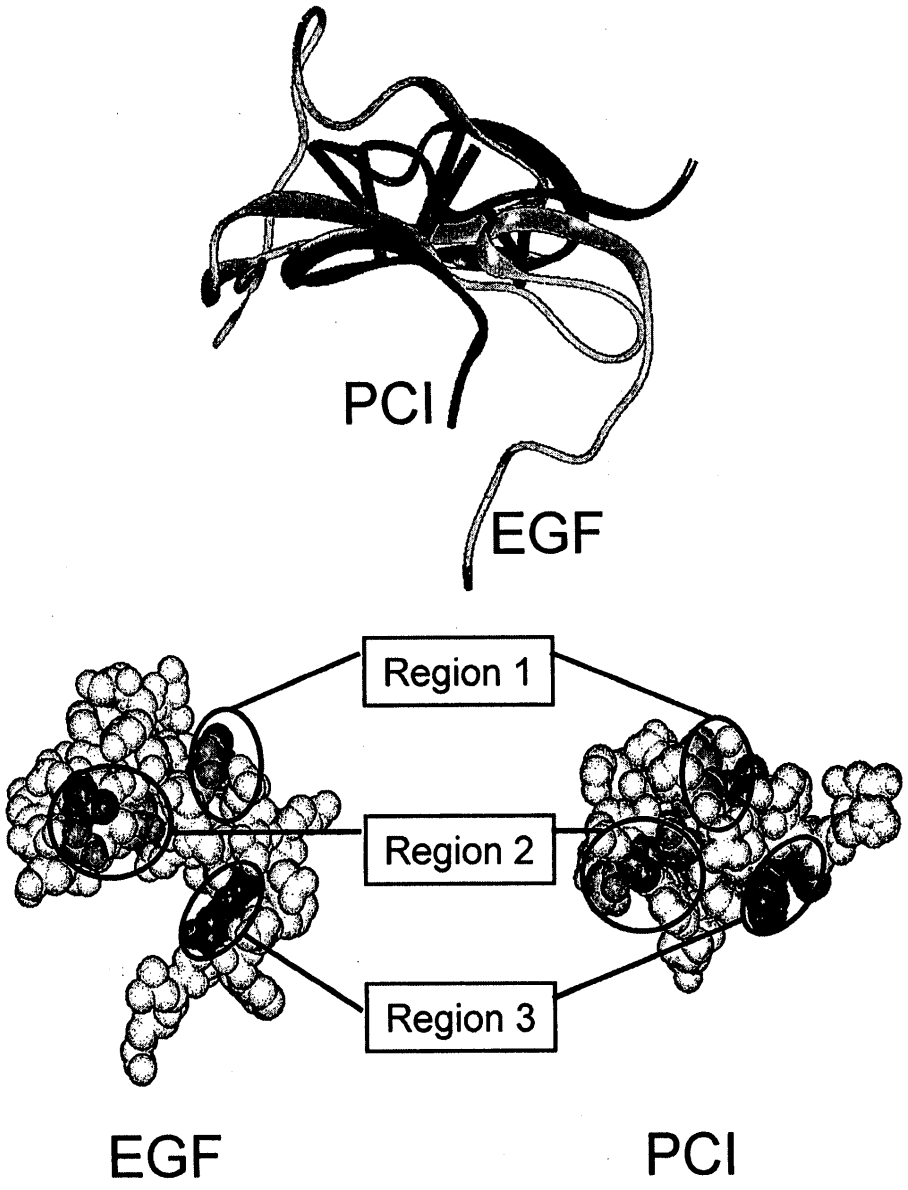

Fig. 4. Structural comparison between PCI (red) and EGF (green) structures. The α carbon chains are superimposed by their disulphide bridge topology. The Van der Waals surface representation shows equivalent residues colored according to their physicochemical properties: magenta for aromatic, green for polar, blue for basic and orange for non-polar residues.

lacks any regular secondary structure, except for a short internal five-residue helix. PCI belongs to the cystine-knot or T-knot super-family of proteins, named for their particular pattern of disulphide bridges [92]. From the 27-residue globular core of PCI, a seven residue N-terminal and five residue C-terminal tail protrude. No functional role has so far been assigned to the N-terminal tail (residues 1–6), whose conformation seems to be undefined. The C-terminus docks into the active site of carboxypeptidase, leading to a stopper-like inhibition mechanism. The inhibitor behaves as a tightly bound peptide

product. Its C-terminal residue (Gly-39) is cleaved by the enzyme and the rest of the inhibitor remains bound to the enzyme by extensive contacts [20]. The functional importance of the tail (residues 35–39) as the primary contact site to the enzyme, and of a short stretch of residues of the core, the secondary binding site (residues 28–31) located around Trp-28, have been experimentally proved by chemical modification studies [93]. Both regions have been shown to be essential in binding. In fact, all residues in the C-terminal tail establish contacts with CPA except Gly-35 [20].

Protein engineering is currently being used by our research group to analyze the structure-function relationship in PCI and to redesign it for biotechnological purposes. The small size of the PCI core and the high number of conformational constraints it contains (three disulphides in 27 residues) constitute a serious challenge for such a redesign. Mutagenesis in the core easily perturbs the disulphide pairings and the native conformation of the protein. In fact, PCI is a good model for studying the problems associated with the engineering of small disulphide-rich proteins. In contrast, its small size and internal rigidity facilitate the computational simulation of protein redesign by molecular dynamic (MD) approaches.

We have cloned and overexpressed, as a soluble protein, a synthetic gene for PCI [94,95], and more recently the wild type gene [96]. A series of PCI mutant forms were designed and expressed and their inhibition constants for CPA measured. The specific mutants, either by replacement or by truncation, were chosen according to: (a) mutants of residues likely to be involved in the interaction with carboxypeptidase and, (b) mutants putatively related to the core stability and folding of the PCI molecule. Among the most informative mutants are: P36G; Y37F; Y37G; V38A; V38G; V38I; V38A; V38F; V38L; G39F; G35P+P36G;Y37G+delG39; delG39; delV38G39; delY37V38G39; delP36Y37V38G39 [97].

Summarizing the results obtained from the above site directed mutants has allowed the identification of the key residues for PCI-CPA interaction in the PCI tail and the estimation of the energetic contribution of their chemical groups to the binding to CPA [97]. From the above mutant series, Val-38 was deduced to be a key residue: its side-chain hydrophobic contribution was very important for the stability of the protease-inhibitor complex (about 50% of the ΔG). The analyses determined that the deletion mutant delV38G39 has an inhibition constant for CPA 10^4-fold higher than that of wild type PCI (wtPCI). The interactions of V38 with CPA residues contribute in 5.4–5.7 kcal mol^{-1} to the overall stability of CPA-PCI complex (11.9–12.1 kcal mol^{-1}). Two of the mutants with smaller side chains, V38G and V38A, allowed the estimation that the contribution of the three side-chain aliphatic groups of valine 38 in the overall stability of the complex is 3.4–4 kcal mol^{-1}. Another two mutants with larger side chains, V38L and V38I, were constructed, the first one being a significantly worse inhibitor than the wild type. These results suggest that only aliphatic groups in positions β and γ of residue 38 of PCI - but not those in δ - can establish van der Waals interactions with atoms of the active center of CPA and participate in binding. The energetic contribution of each methyl/methylene group in those positions can be estimated as 1–1.5 kcal mol^{-1}. This hypothesis was strongly reinforced by computer simulation analysis. The above mentioned mutations of residue 38 only affected the hydrophobicity and volume of its side chain. Therefore, we assume that the entropic factors of the interaction between PCI and CPA were those that suffered most from the perturbation. The changes in volume were also compared with the variation in hydrophobicity, that is, with the tendency of the side chain to become buried. A linear relationship was found between $\Delta(\Delta G)$ of the complexes calculated from the experimental data and the increase in the excluded volume for the different PCI variants. All these results strongly support the hypothesis that no side chain aliphatic Cδ in position 38 is buried in the active site cavity of CPA, probably being in contact with the water shell of the complex and not making any contribution to its stability. The S1 subsite position in the enzyme, which is located in a narrow passage close to residue 38 of PCI, is followed by a wide pocket. Only chemical groups in positions β and γ of residue 38 would be in appropriate positions to be buried in the active center of the CPA, while Cδ in mutants V38I and V38L are faced to the water shell. In the case of mutant V38L, one of the γ methyl groups of Val-38 is absent. In wtPCI, this γ methyl interacts with the aromatic rings

of two CPA residues. In the case of mutant V38I, in which both γ methyl groups are present, the K_i was similar to that of wtPCI. The δ methyl group of the mutant is directed backwards to the PCI core, specifically to Trp-28 of PCI, and has no significant contacts with CPA. Therefore, it cannot contribute to the stability of the complex [97].

The C-terminal tail residues that are closer to the PCI core seem to play a role in the proper positioning of the tail for the most efficient binding to the enzyme. Thus, Gly-35 backbone atoms establish a double hydrogen bond with Ala-26 of the core. In this context, it is worth mentioning that P36G mutant presented a lower rate of association of the inhibitor to the enzyme. Computer analysis by molecular dynamics (MD) suggests this change to be the consequence of the higher mobility of the PCI C-tail caused by the replacement. The difference between the kinetic constants of a synthetic peptide with the sequence of the five C-terminal residues of PCI (estimated *Ki* about 1 mM), and those of the mutant P36G-PCI reflect the importance of the conformational rigidity of the C-tail of PCI on its inhibitory ability. Tyr-37 also contributes to such a proper positioning. Mutant Y37G shows an increase in *Ki*, which allowed to estimate that the contribution of the aromatic ring of this residue to the overall stability of the protein-inhibitor complex is about 0.5 kcal mol^{-1}. This mutant also showed a biphasic behavior, becoming a significantly worse inhibitor after the C-terminal glycine residue was cleaved by CPA (C. Marino, E. Querol and F.X. Avilés, unpublished data). Finally, the kinetic constants of deletion mutants of PCI, show the remarkable fact that two of them, delV38G39 and delY37V38G39 appear to have the same binding energy. We interpreted this as a result of the increased mobility of the C-tail when Val-38 is absent, and the subsequent disruption of the interactions of Tyr-37 with CPA residues.

To gain insight into the essential elements of the structure, and to facilitate its redesign, the folding and unfolding pathways of PCI has also been investigated [98]. Stop/go experiments of both acid and iodoacetate-trapped intermediates, followed by RP-HPLC have been used for such purpose. The folding of PCI proceeds through an initial stage of non-specific disulphide formation (packing), followed by disulphide reshuffling (consolidation) of partially packed intermediates to acquire the native structure. The process of non-specific packing involves a sequential flow of fully reduced PCI through 1- and 2-disulphide intermediates and leads to the formation of scrambled 3-disulphide species. All three classes of intermediates are highly heterogeneous. Disulphide reshuffling occurs at the final stage which refines and consolidates the scrambled species to acquire the native conformation. The efficiencies of disulphide formation and disulphide reshuffling can be selectively regulated by redox potential. Disulphide formation is promoted by cystine or oxidized glutathione, whereas disulphide reshuffling requires free thiols, such as cysteine, reduced glutathione or β-mecaptoethanol. Consolidation of scrambled species to form the native PCI represents the major rate-limiting step. PCI refolds at different times (from minutes to hours), but when folding of PCI was carried out in the presence of 2 mM cystine, more than 98% of the intermediates accumulate as the scrambled species after 1 min of folding. Furthermore, denaturants (5 M GdmCl or 8 M urea) mainly disrupt the final stage of PCI folding and exert no apparent influence on the early stage of non-specific packing [98]. The folding of the PCI was also computationally simulated by MD in explicit solvent, although native disulphide bond constraints were introduced. Native structures were recovered in most simulations [99].

Recently we have cloned the PCI native gene from a cDNA library from mRNA of abcisic acid treated potato leaves [96]. The 102 residue sequence shows a 29-residue N-terminal signal peptide, a 27-residue N-terminal pro-region, the 39-residue mature PCI protein and a 7-residue C-terminal extension. The cDNA and amino acid sequences are similar to the tomato homologous form [100], except for a short insertion in the potato cDNA at the pro-region. Northern blot analysis demonstrates that the PCI gene is transcriptionally activated by wounding, and wound signaling can be induced by abcisic or jasmonic acids. The protein accumulates within vacuoles. The inability of the pro-form to inhibit carboxypeptidases, and its stability to carboxypeptidase digestion, suggest that the C-terminal pro-domain may have, besides a probable vacuolar sorting function as previously suggested for the tomato isoform [100], a role in the modulation of the inhibitory activity of PCI.

An interesting result recently found is that PCI behaves as an EGF antagonist, the first antagonist of human EGF ever reported, therefore being a putative antitumor agent [101]. It is known that EGF and its receptor (EGFR) are involved in many aspects of the development of carcinomas, including tumor cell growth, vascularization, invasiveness and metastasis. As EGFR has been found to be overexpressed in many tumors of epithelial origin, it is a potential target for antitumor therapy. It has been shown that PCI competes with EGF for binding to EGFR and inhibits EGFR activation and cell proliferation induced by this growth factor. PCI suppresses the growth of several human pancreatic adenocarcinoma cell lines, both in vitro and in nude mice. Although several protease inhibitors have been previously reported as potential antitumoral agents [102], this is the first case reported where the effect is not a result of its protease inhibitory activity but of its behavior as an EGF antagonist. Fundamental for the understanding of this observation has been the computer program 'KnotMatch' that we have developed for the superimposition of proteins based on their disulphide bridge topology [103]. This new method for comparing protein structures permits superimposition of two apparently unrelated proteins rich in disulphides, allowing the detection of similar spatial positioning of residues or secondary structures and unexpected relationships between non-homologous or remote homologous proteins can be disclosed. As mentioned above, PCI has a special disulphide scaffold called a T-knot, that is also present in several growth factors including EGF and TGFα. According to such a comparative approach, PCI shows structural similarities with these growth factors (Fig. 4), a fact that can explain the antagonistic effect of the former.

3.2. Other metallocarboxypeptidase inhibitors

3.2.1. Tomato carboxypeptidase inhibitor

Tomatos contain a metallocarboxypeptidase inhibitor (MCPI) with an amino acid sequence highly similar to that of PCI (70% identity at the protein level, 85% at DNA level, and strong immunological cross-reactivity). Wounding of the tomato leaves causes a 100-fold increase in MCPI RNA levels without a concomitant increase in MCPI protein level [100]. The function of potato and tomato inhibitors is probably related to plant defense mechanisms [88].

3.2.2. Ascaris suum carboxypeptidase inhibitor

A. suum is an intestinal parasite that produces several protease inhibitors. Among them an inhibitor of carboxypeptidases A and B has been described [89,104]. This inhibitor would present a typical stoichiometry of two molecules of inhibitor to one molecule of enzyme. The carboxypeptidase inhibitor is a 65- or 66-amino acid residues polypeptide (MW 8 kDa) whose sequence displays some similarity with inhibitors from *Solanacea* at a short 9-residue internal stretch and at the C-terminal 5 residues [89].

3.2.3. Medical leech carboxypeptidase inhibitor

We have recently isolated a carboxypeptidase inhibitor from the medical leech *Hirudo medicinalis* [90]. The cDNA encodes a 66-amino acid residues polypeptide. It does not show sequence similarity to any other protein except at its C-terminal region where a certain similarity is found with inhibitors from *Solanacea*, suggesting a similar mechanism of inhibition. It is a tight-binding, competitive inhibitor for different types of pancreatic-like carboxypeptidases. The resolution of its three dimensional structure by X-ray crystallography and NMR is under way.

3.2.4. Rat brain carboxypeptidase inhibitor or tissue carboxypeptidase inhibitor (TCI)

This is the largest proteinaceous carboxypeptidase inhibitor reported. The cDNA encodes a 233-amino acid residues (MW 26 kDa) protein present in non-pancreatic tissues (e.g., brain, lung, digestive tract). Its sequence does not present significant homology with the rest of reported carboxypeptidase inhibitors other than a limited degree of similarity between a short segment of its sequence and the activation segment of porcine procarboxypeptidase B. TCI is a hardly reversible, non-competitive and potent inhibitor of CPA1, CPA2 and mast-cell CPA. It lacks a signal peptide, a fact that suggests a cytosolic localization. Therefore a rather general functional role, such as the control of cytosolic protein degradation was suggested [105].

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